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<p>14. ABSTRACT: Currently there is no good therapy for two subtypes of human breast cancers (BC) which have aggressive disease phenotype, often resulting in high mortality. They include the triple negative BC (TNBC), and the endocrine resistant Luminal B subtypes. Our goal in this project is to develop a new class of drugs targeted for these hard-to-treat tumors which is safe and effective. Towards that goal we have identified a novel target called Separase, an enzyme important for cell division. Separase is an oncogene which is overexpressed in >60% of BC, 50% of TNBC, and 65% of Luminal-B tumors, and its overexpression strongly correlates with high incidence of relapse, metastasis, and a lower 5-year overall survival rate. We hypothesize that modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting resistant, Separase-overexpressing tumors, particularly the hard-to-treat TNBC. Using a high throughput screen, we have identified a novel small molecular inhibitor of Separase, named Sepin-1. The goal of this project has been, 1) to examine pharmacokinetics, pharmacodynamics, and the efficacy of Sepin-1 in animal models, and 2) to characterize the mechanisms of Sepin-1 action at the molecular level. This is the 1st annual progress report for this project.</p>				
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June 10, 2016

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SUBJECT: Annual Report for Grant Number W81XWH-15-1-0122

Dear Commander:

Please find enclosed the first annual progress report for the grant entitled “Development of a Novel Separase Inhibitor, Sepin-1 for Breast Cancer Therapy” for the period of May15, 2015 - May 14, 2016. The report has been prepared in accordance with the guidelines provided in the memo dated April 15, 2016.

If any further questions or points of clarification regarding this report arise, please contact me at 832-824-4575 or by E-Mail: pati@bcm.tmc.edu. or Fax: 832-825-4651.

Yours sincerely,



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Introduction

Current treatment options for advanced, metastatic, highly heterogeneous, and refractory breast cancer (BC) tumors are extremely limited, contributing to most of the BC-related deaths in USA and around the world primarily due to drug-resistance, and drug-related side effects and toxicities.¹⁻³ The goal of this project is to develop a new class of drugs for the treatment of refractory BC that are safe and effective with the least side effects.

Separase, an enzyme that cleaves the chromosomal cohesin complex during mitosis, is overexpressed in more than 60% of BC and 50% of TNBC, and 65% of Luminal-B BC tumors and its overexpression strongly correlates with aneuploidy, high incidence of relapse, metastasis, and a lower 5-year overall survival rate.⁴⁻⁸ Separase is an oncogene,^{6,7} and overexpression of Separase induces aneuploidy, genomic instability, mammary tumorigenesis, and intratumoral heterogeneity in mice.^{4,6} We hypothesize that modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting resistant, Separase-overexpressing aneuploid tumors, particularly the hard-to-treat TNBC.⁹

We hypothesize that pharmacologic modulation of Separase enzymatic activity constitutes a new therapeutic strategy for targeting Separase-overexpressing aneuploid heterogeneous tumors. We further hypothesize that by decreasing Separase activity to a therapeutically useful degree of inhibition by Sepin compounds, we will effectively reprogram the Separase-overexpressing tumors and that partial Separase inhibition will selectively eliminate Separase-overexpressing tumor cells addicted to elevated Separase expression while sparing normal cells, and thereby preventing aneuploidy and aneuploidy-associated tumor heterogeneity.

Following is the annual progress report for the first year of this project. We have made substantial progress on all our technical objectives. Our results indicate that Sepin-1 is not only effective in selectively killing Separase overexpressed tumor cells but also synergistic with the existing the chemotherapies including Tamoxifen and Carboplatin.

Key Words

Separase, Separase Inhibitors, Sepin-1, Triple Negative Breast Cancer

Accomplishments

The specific aim for this project is to evaluate Sepin-1 for breast cancer therapeutics *in vivo*. The technical objectives are: 1) to evaluate the efficacy of Sepin-1, and its analogs in Separase-overexpressed tumors, and 2) to characterize the mechanisms of Sepin action in inhibiting tumor growth. Our strategies here are twofold: a) to characterize the activity of Sepin-1 towards validating it as a BC therapeutic *in vivo*, and b) to assay the tumor-regressing activity of Sepin-1 and in combination with the existing breast cancer chemotherapy in *human* TNBC xenograft models. Experimental approaches are to determine the pharmacokinetics, toxicity, and efficacy of Sepins as an inhibitor of BC progression. Following is a list of accomplishments in the year-1 of this project.

- Sepin-1 formulation has been determined (90% Phosphate Buffered Saline containing 0.1% Tween 20 (PBST) and 10% DMSO) for Intra peritoneal injection (IP) and oral gavage.
- A method to analyze the pharmacokinetics of Sepin-1 has been developed. The standard curve has been set up with Sepin-1 in PBS. A bioanalytical method to detect Sepin-1 in plasma has been developed and validated.
- Appropriate route of administration and dose, frequency of dosage, and bioavailability of Sepin-1 in SCID-beige mice have been worked out. Both IP (5-10mg/Kg/daily) and oral gavage (30-40mg/kg) can be safely used. Sepin-1 was administered to mice with four time a week (Monday, Tuesday, Thursday and Friday).
- We have developed three breast cancer cell line models (MDA-MB-468, BT-474, and MCF7)-transduced with lentivirus containing pCMV-Luciferase-EF1a-copGFP-2A-puro. Tumors derived from these cell lines in mice can be visualized and monitored using luciferase signals. These mice will be used to study the effect of Sepin-1 in tumor progression.
- Patient-derived TNBC xenograft studies to examine the efficacy of Sepin-1 is in progress. We have established two patient-derived mammary tumors in SCID-beige mice and 10 more are currently in progress. Two xenograft models were treated with Sepin-1, toxicity, mammary tumor progression and metastasis were assayed.
- We have examined the effect of Sepin-1 in combination with other anti-BC therapies using breast cancer tissue culture models ex vivo. We have tested the synergistic effect of Sepin-1 and four drugs including Tamoxifen, Docetaxel, Doxorubicin and Carboplatin in BT-474 (ER⁺ PR⁺ HER⁺), MCF7 (ER⁺ PR^{+/−} HER[−]) and MDA-MB-468 (triple negative) cells. The synergy of Sepin-1 and Docetaxel was found in all three cell lines, while that of Sepin-1 and Doxorubicin was detected in MAD-MB-468 and BT-474 cells, but not in MCF7 cells. No synergistic effect between Sepin-1 and Tamoxifen or Carboplatin were observed in all three cell lines.
- We have established two patient-derived TNBC tumors (MC1 and 4195-TG3 with high and low Separase expression, respectively) in SCID-beige mice and 10 more are currently in progress. MC1 and 4195-TG3 xenografts were treated with various concentrations of Sepin-1 to assay toxicity and to quantify mammary tumor progression. While there is a significant reduction in tumor volume in Separase high MC1 tumors, no significant difference was found among the treatments (control and Sepin-1 1, 5 and 10mg/kg IP) in Separase low 4195-TG3 xenograft confirming our previous result indicating the specificity of Sepin-1 in attacking Separase-overexpressing tumor cells (Zhang et al, 2014).⁹
- We organized a collaborator's retreat named "Step in to Sepin" on April 22, 2016 to discuss our progress over the past year and chalk a plan for the second year of the project. This was attended not only by our collaborators but also the patient advocates including *Mrs. Anne Meyn* and several companies who is interested in the preclinical development of Sepin-1. Around 50 people attended this retreat and we had a number of suggestions and recommendations that we are currently pursuing to complete the preclinical IND-enabling studies for Sepin-1.

Following is some of the detailed results from our studies per the proposed statement of work:

Task 1: To investigate the pharmacokinetics (PK) and pharmacodynamics (PD) of Sepin compounds. (1-24 months)

Progress:

We have developed a Liquid chromatography–mass spectrometry (LC-MS) method to measure Sepin-1 in mouse, rat and dog plasma. The method includes 1) understanding of Sepin-1 behavior in terms of the physicochemical properties such as chemical structure, functional group(s), molecular weight, solubility, and stability in formulation. 2) MS/MS (MRM) scanning and optimization including Electron Spray Ionization (ESI). 3) Development and optimization of the LC method including reversed-phase using a UPLC BEH C18 column, mobile phase selection and optimization (choosing best buffers, pH, flow rate), and selection of the best internal standard, and 4) Development of sample preparation method and optimization, a most critical step to minimize ion suppression/ion enhancement for reliable MS detection and to optimize Protein precipitation techniques (PPT) and Solid-phase extraction (SPE).

Stability studies were carried out in different solvents. Sepin-1 is highly stable in water with one observed peak. In Phosphate Buffered Saline (PBS) however, two MS peaks (negative mode) were observed. In organic solvents, degradation of Sepin-1 over time was noted. Importantly, when Sepin-1 spiked into plasma, the MS signal was weak and disappeared over time. By basifying PBS or plasma, one Sepin-1 peak is detected in MS positive mode, which ionizes better in basic than in an acidic environment.

Two different **Mobile Buffer Systems** were explored for chromatography optimization (Acidic and Basic). Acidic mobile buffer system included weak signal in neutral pH. However, changing pH of sample solution improved response. Using basic mobile buffer system one distinct MS peak was obtained, where analyte response increase over time and basic sample solution at pH 10, stabilizes Sepin-1 response.

Liquid Chromatography parameters were worked out using an Acquity Ultra Performance Liquid Chromatography (UPLC) System from Waters with a BEH C18, 50 x 2.1 mm, 1.7 µm column. Basic buffer system included Mobile Phase A: 6.5 mM Ammonium Bicarbonate, pH 8.30 and Mobile Phase B: 6.5 mM Ammonium Bicarbonate in 95% Methanol. Flow rate of 0.5 mL/min and column temperature 40°C was maintained. A chemically similar to drug analyte named Sepin-1.11 was used as an internal standard which behaves similarly as Sepin-1 in basic LC buffer system. Representative calibration curve, quadratic fit ($1/x^2$ weighing) were obtained. Using this method nominal concentration of Sepin-1 from 3 to 400 ng/mL of plasma can be detected.

3) Route of administration and dose, frequency of dosage.

Progress:

Both IP (5-10mg/Kg/daily) and oral gavage (30-40mg/kg) can be used. Sepin-1 was administered safely without any observable effect to mice with four time a week (Monday, Tuesday, Thursday and Friday).

Task 2: To assay a therapeutic role for Sepins in Separase-overexpressing breast cancer (BC). (6-30 Months)

a) Establishment of mammary tumors (MT): (GFP)-tagged MDA-MB-468 (basal-like; ER/PR/HER2 triple-negative), BT474 (luminal-B, ER⁺, PR[±], HER⁺), and MCF7 (luminal-A, ER⁺, PR[±], HER2-) human BC cells will be injected into the inguinal (#4) mammary fat pad of female SCID-beige mice.

Progress:

We have developed three cell lines that were transduced with lentivirus containing pCMV-Luciferase-EF1a-copGFP-2A-puro for MDA-MB-468, BT-474, and MCF7 lines. Tumors derived from these cell lines can be monitored using luciferase signals.

b) Sepin treatment: One to two weeks after tumor cells are inoculated, when MTs are ~200 mm³ in size, the mice will be treated with vehicle (PBS) or Sepins (5, 50, or 75 mg/kg BW or concentrations determined from Task-1) by IP 5X per week for a maximum of 12 weeks. (6-14 months)

Progress:

We have tested the effect of Sepin-1 on the tumor growth after the cells of MCF7 transduced pCMV-Luciferase-EF1a-copGFP-2A-puro were xenografted to female SCID-beige mice. Sepin-1 was administered via intraperitoneal (IP) or oral gavage (OG) with four times per week (Monday, Tuesday, Thursday and Friday) for three weeks.

c) Toxicity assessment: Toxicity will be assessed 5X a week by visual evaluation of feeding and behavioral abnormalities, loss of appetite, weight, and other factors. Survival will be monitored and potential LD₅₀ calculated. After necropsy, kidneys and livers will be harvested and weighed (organ weight as % of total BW). Livers will be frozen and hepatotoxicity assessed by activities of liver enzymes. (6-20 months)

Progress:

Significant weight loss was observed in the IP group with Sepin-1 with ≥ 10mg/kg and the Oral Gavage group with ≥ 50 mg/kg, indicating toxicity.

d) Quantification of MT progression: GFP-tagged MT growth will be quantified by analysis of integrated density and area of fluorescence from images acquired using a whole body imaging system. (6-30 months)

Progress:

The luciferase signal was reduced > 49% in IP group with 5mg/kg of Sepin-1 treatment, and about 28% in OG group with 30mg/kg of Sepin-1 treatment, suggesting tumor growth was inhibited.

Task3: Drug combination experiments: Effect of Separase inhibitors in combination with other anti-BC therapies will be examined. (*Months 24-36*)

Progress:

We have tested the synergistic effect of Sepin-1 and four drugs including Tamoxifen, Docetaxel, Doxorubicin and Carboplatin in BT-474 (ER⁺ PR⁺ HER⁺), MCF7 (ER⁺ PR⁺⁻ HER⁻) and MDA-MB-468 (triple negative) cells. Data were analyzed with CompuSyn. We did not observe the synergistic effect between Sepin-1 and Tamoxifen or Carboplatin in all three cell lines. The synergy of Sepin-1 and Docetaxel was found in all three cell lines, while that of Sepin-1 and Doxorubicin was detected in MAD-MB-468 and BT-474 cells, but not in MCF7 cells.

Task 4: Effects of Separase inhibitors *in vivo* using a four-arm (Vehicle, Sepin-1, and 2 promising Sepin analogs) mouse clinical trial, using 38 TNBC tumor xenograft lines. (*Months 1-36*)

a) **Transplantation of xenograft models.** 38 xenograft lines will be transplanted at 2- to 3-week intervals, two xenograft lines per interval, in preparation for treatment studies based on the growth kinetics of each model.

Progress:

Two patient-derived xenografts MC1 and 4195-TG3 have transplanted and used for Sepin-1 dose range finding studies.

b) **Treatment of xenograft models.** When tumors reach the appropriate size, mice will be randomized to treatment groups, and treated. Tumor growth will be monitored during the treatment period.

Progress:

No significant difference was found among the treatments (control and Sepin-1 30, 35 and 60mg/kg IP) in 4195-TG3 lines. However, Sepin-1 treatment significantly curtailed the growth of MC1 xenograft.

c) Tumor harvest. At end of treatment, residual tumors will be harvested and processed for downstream assays to determine the effects of treatment.

Progress:

Tumor tissue has been collected from all xenograft studies to date. Moving forward, we will also collect plasma in order to do PK/PD studies.

Task 5: To characterize the mechanisms of Sepin action in inhibiting tumor growth: (*9-30 months*)

Progress:

Normal mammary epithelial cells from mouse were treated with 7.5 μ M of Sepin1 for 0, 4, 12 and 24h (duplicates). The cells were harvested and total RNA was extracted using RNeasy Plus Mini Kit (Qiagen). The RNA samples were analyzed using Agilent (SurePrint G3 Mouse GE 8x60k) microarray. The data is currently being analyzed to examine the effect of Sepin-1 in gene expression and to identify Sepin-1 effect on any specific pathway.

Reportable Outcomes

Manuscript:

Do Ha T, Zhang N., **Pati D***, Gilbertson SR*. Synthesis and activity of Benzimidazole-1,3-dioxide inhibitors of Separase (*co-corresponding author). **Submitted** (See Appendix-II)

Abstract:

Zhang N., Do Ha, Scorsone K., Mukherjee M, Li X-N, Sumazin, P, Ahmed N, Berg S, Gilbertson, SR, **Pati D**. Sepin-1, a Novel Separase Inhibitor, for Breast Cancer Therapy. Drug Discovery & Therapy World Congress, 2015, Boston, MA, July 22-25. **Selected for Platform Presentation**

Zhang N., Do H., Demerzhan S., Dobrolecki L., Lewis M., Berg S., Gilbertson S., **Pati, D.** Inhibitor of Cohesin-Protease, Separase, as Novel Agents for Breast Cancer Therapy. Innovations in Cancer Prevention and Research Conference. November 9-10, 2015, Austin TX Program and Abstract Book Abstract#407

Zhang Nenggang, Do Ha, Dobrolecki Lacey E., Lewis Michael T., Berg Stacey, Gilbertson Scott R., and **Pati Debananda**. Separase Inhibitors for Cancer Therapy. 11th Annual Texas Children's Cancer Center Research Symposium, May 14, 2015, Houston, TX. **Poster Presentation**

Employment:

Following personnel worked for the first year of this project. Dr. Debananda Pati – 30% entire period, Dr. Mariya Morar – 100% 9/14/15 – 1/25/16; Dr. Samridhdi Paudyal – 100% 11/30/15 – 2/29/16, Dr. Eliza Ruben – 100% 3/14/16 – 4/29/16; Dr. Michael Lewis – 5% entire period, Lacey Dobrolecki – 15% entire period. A summer studentship has been granted to Mr. Roland Brutus, an undergraduate student from Texas Southern University, Houston, TX by the SMART program of Baylor College of Medicine to work on this project. SMART program is funded by the National Institute of Health, National Institute of General Medical sciences. *Mrs. Anne Meyn, a project LEAD graduate with seven year experience as an active breast cancer research advocate serves as an honorary members for this project.*

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Impact

Therapeutic treatment options for patients with stage TNBC are very limited and often unsuccessful. There are no targeted agents to supplement cytotoxic chemotherapy. The TNBC phenotype is impervious to therapies commonly used in other breast cancer subtypes, including hormonal therapy and Her-2 receptor antagonism. It is established that in the absence of ER, PR and HER-2, endocrine therapies such as Tamoxifen and aromatase inhibitors and HER-2 directed therapies such as Trastuzumab and Lapatinib are not efficacious. We are seeking a new drug applications (NDA) for Sepin-1, an inhibitor of Separase, as a therapeutic target for treatment of patients with hormone refractory breast cancer who have previously received at least two chemotherapeutic regimens for metastatic disease. We believe Sepin-1 would be superior over current therapies for the following reasons.

- TNBC is a heterogeneous disease comprising a spectrum of cancers with distinct activated biological pathways. Overexpression of Separase is a fundamental characteristic shared across all TNBC and Separase is a novel target.
- Alternate targeted approaches currently being explored, including PARPi, EGFR inhibitors, antiangiogenic agents, and checkpoint kinase 1 inhibitors have not made a substantial impact on clinical outcomes in metastatic TNBC to date.
- Relatively low cost, straight forward CMC, and manufacturing scalability compared to monoclonal antibody and immunotherapy approaches.
- Separase expression is very high in 25-50% TNBC.
- Small molecule, relatively simple and low cost synthesis.
- Targeted inhibition of a TNBC applicable pathway separate from existing standard of care Mechanism of Action
- Relatively low toxicity.
- Compound amenable to refinement of formulation for future development and extended patent protection (liposomal/pegylation/etc.).

IND-enabling studies for Sepin-1 is funded through a product development grant from CPRIT and the mechanisms of Sepin-1 action is pursued through this DOD grant.

Changes/Problems

This project was slow to start due to personnel issues. It took us almost 6 months to hire a suitable person (Dr. Maria Morar) but within three months she has to leave because of her family relocation to California. We had only one person Dr. Samridhdi Paudyal who worked part time until February, 2016. We are currently working to fill this position. However, with this limitation we have made significant progress in this project.

Products:

Not Applicable

Participants & Other Collaborating Organizations:

Not Applicable

Special Reporting Requirements

Not Applicable

Appendix-I

Statement of Work (Work accomplished)

Month	Technical Objectives	Status
1-24	Investigate the pharmacokinetics (PK) and pharmacodynamics (PD) of Sepin-1	In Progress
	<i>Milestones:</i> PK method has been developed. Appropriate route of administration and dose, frequency of dosage, has been determined. Bioavailability of Sepin-1 in SCID-beige mice will investigated in Yr. 2.	
6-30	To assay a therapeutic role for Sepin-1 in Separase-overexpressing breast cancer (BC).	In Progress
	<i>Milestones:</i> Toxic effect of the Sepin-1 has been investigated. More detailed studies are ongoing. Oncostatic effect of Sepin-1 on tumor regression are currently being evaluated.	
1. 24-36	Drug combination experiments: Effect of Separase inhibitors in combination with other anti-BC therapies	Started
	<i>Milestones:</i> 1) Safety profile of the combination, including the maximum tolerable dose (MTD) and the dose-limiting toxicities (DLTs) will be determined. Not attempted 2) Anti-tumor activity, pharmacodynamics (PD), PK will be assayed. Started 3) Modeling of drug interactions will be performed. In Progress	
1-36	Effects of Separase inhibitors <i>in vivo</i> using a four-arm (Vehicle, Sepin-1, and 2 promising Sepin analogs) mouse clinical trial, using 38 TNBC tumor xenograft lines.	In Progress
9-36	To characterize the mechanisms of Sepin action in inhibiting tumor growth	Started

APPENDIX-II

Submitted Manuscript

Synthesis and Activity of Benzimidazole-1,3-dioxide Inhibitors of Separase

Ha T. Do,[#] Nenggang Zhang,[#] Debananda Pati,^{‡*} and Scott R. Gilbertson^{†*}.

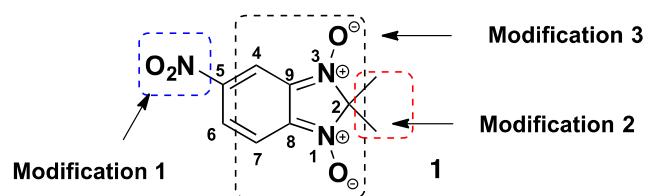
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ABSTRACT: Due to the oncogenic activity of cohesin protease, Separase in human cancer cells, modulation of Separase enzymatic activity could constitute a new therapeutic strategy for targeting resistant, Separase-overexpressing aneuploid tumors. Herein, we report the synthesis, structural information, and structure-activity relationship (SAR) of Separase inhibitors based on modification of the lead molecule 2,2-dimethyl-5-nitro-2H-benzimidazole-1,3-dioxide, named Sepin-1, (**1**) identified from a high-throughput-screen. Replacement of NO_2 at C5 with other functional groups reduce the inhibitory activity in Separase enzymatic assay. Substitution of the two methyl groups with other alkyl chains at the C2 moderately improves the effects on the inhibitory activity of those compounds. Modifications on 2*H*-benzimidazole-1,3-dioxide or the skeleton have variable effect on inhibition of Separase enzymatic activity. Density-functional theory (DFT) calculations suggest there may be a correlation between the charges on the oxide moieties on compound **1** and its activity in inhibiting Separase enzyme.

INTRODUCTION

In recent years, breast cancer (BC) has become one of the most common types of cancer in humans, both in new cases and cancer death rates in females.¹ Separase, an enzyme that cleaves the chromosomal cohesin complex during mitosis, is overexpressed and mislocalized in a number of human tumors,² overexpressed in more than 60% of BC and 50% of triple-negative BC, and 65% of Luminal-B BC tumors.^{2,3} Separase overexpression in animal models induce aneuploid mammary tumors. Since only recently structural information about Separase has become available,⁴ high throughput screening was used to search for potential Separase inhibitors.⁵ The screening of 14,400-compound library provided 5 compounds with good activity, in which 2,2-dimethyl-5-nitro-2*H*-benzimidazole-1,3-dioxide (**1** Figure 1) exhibited the highest activity toward inhibiting Separase with a half-maximal inhibitory concentration (IC_{50}) of 15 μM .⁵ In the original paper this compound was named Sepin-1.⁵ Further studies showed that the benzimidazole-1,3-dioxide **1** inhibits the growth of Separase-overexpressing human triple-negative BC xenografts in mice in a dose-dependent manner, and has no appreciable effect on such tumors with low-Separase expression, suggesting that the specificity and efficacy of this compound in targeting tumors is related to Separase overexpression. Targeting Separase by **1** also results in high levels of apoptosis. These results suggest that inhibition of Separase represents a new line of therapy to treat breast and other tumors overexpressing Separase. In this contribution, we report the synthesis, structural information, biological activity, and structure-activity relationship (SAR) study of Separase inhibitors



based on structural modification of Sepin-1 (**1**). The study was designed to determine the sites on the molecule most relevant for activity and through their modification develop more active analogs. Three regions of **1** scaffold were modified and the resulting molecules tested for activity; the substituents at the C5 position, the heterocycle attached to the benzene ring, and the C2 position of carbon between the two nitrogen atoms (Figure 1).

The structure of Sepin-1 (**1**) is not typically what one would consider to be “drug like”. The molecule is small, highly polar, possess a nitro group and may be redox active. However this report is not the first time these molecules have been found to have selective and interesting biological activity. Gonzalez has previously reported that derivatives of the 2*H*-benzimidazole-1,3-dioxide scaffold are effective anti-trypanosomatid agents. With the tested versions possessing no toxicity in BALB/c mice with dosing of 30 mg/kg/day for 10 days, with the animals observed for 60 days.⁶

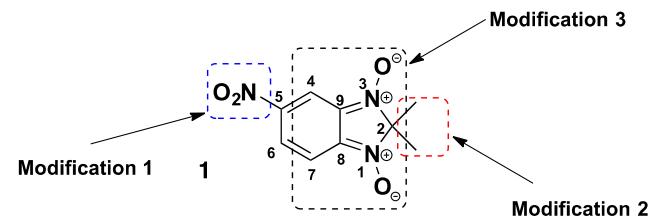


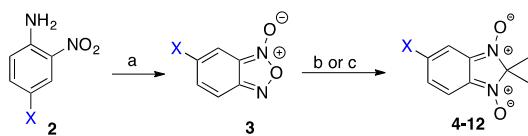
Figure 1. Structure of **1** with domains marked for structure activity study.

RESULTS AND DISCUSSION

Modification of benzimidazole-1,3-dioxide **1**

The general route to the benzimidazole derivatives starts with the acid catalyzed cyclization of nitroaniline (**2**) to give benzofuroxans (**3**). Reaction with an alcohol in the presence of sulfuric acid then provides the *2H*-benzimidazole-1,3-dioxides derivatives (**4-12**).^{7, 6} Versions where the nitro group was replaced by other functionality, including carboxylic acid, ester, fluorine, bromine, and ethoxyl groups, were synthesized by starting with the appropriate nitroaniline (**2**). The bromine-substituted compound (**11**) was used for further modification by the Suzuki coupling reaction. Additionally, the product bearing fluorine (**9**) was reacted with different nucleophiles such as primary amines, allowing for modification at the C₅ position on the *2H*-benzimidazole-1,3-dioxides skeleton. Scheme 1 outlines the synthetic route and the chemical structures of derivatives with the nitro group replaced by other groups to give compounds **4-12**.

Scheme 1. Synthesis of derivatives at C₅ position



a) NaNO_2 , H_2SO_4 , AcOH , NaN_3 , H_2O , $0\text{-}80^\circ\text{C}$ or KOH , EtOH , NaOCl 8%. b) $i\text{PrOH}$, H_2SO_4 , RT, 2 hrs. c) $i\text{PrNO}_2$, pyridine, THF , RT, 18 hrs. d) (**9** and **10**) *n*-butyl amine, EtOH , RT, overnight. e.) (**11** and **12**) PhB(OH)_2 , $(\text{SiPr})\text{Pd}(\text{allyl})\text{Cl}$, *t*-BuONa, 1,4-dioxane, 60°C , 3 hrs

TABLE 1. Yield of derivatives at the nitro group

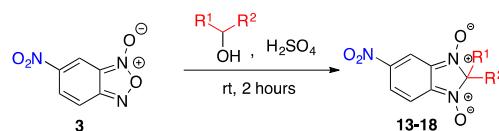
Compound	X	Yield (%) from 3
1	NO_2	74
4	COOH	31
5	COOCH_3	56
6	OEt	38
7	CF_3	49
8	SO_2CF_3	42
9	F	77
10	$\text{NH}(\text{C}_4\text{H}_9)$	70
11	Br	71
12	C_6H_5	31

The modification of functionality at the C₂ position of the *2H*-benzimidazole-1,3-dioxides was obtained by using different secondary alcohols in the reaction with benzofuroxans **3** while keeping the composition of other positions, such as nitro group, the same as in **1**. Because this step in the reaction mechanism involves a carbocation, the products obtained can result from rearrangement. Due to intermediacy of a carbocation and the harsh conditions requiring sulfuric acid the scope of alcohol substrates is limited. The installation of functional groups, including

alkenes, alkynes, alcohols and amines into this position, has been challenging. Six different compounds (**13-18**, Scheme 2) that have alkyl chains, spiro-derivatives, and aryl chains have been synthesized and evaluated to determine the effect of this position on the biological activity of the *2H*-benzimidazole-1,3-dioxides derivatives.

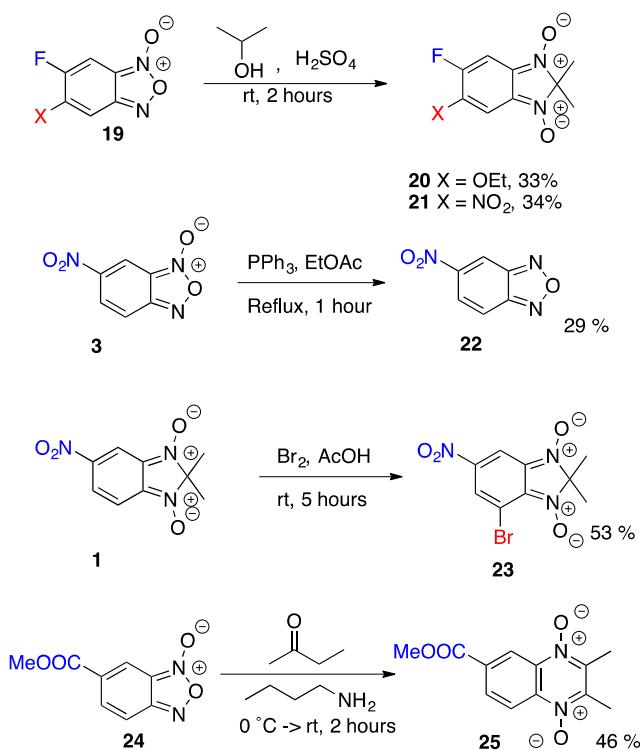
Further modification of **1** was performed in a series of individual reactions. Compounds **20** and **21** were generated from their corresponding benzofuroxan using sulfuric acid and *i*-propanol. Structure **23** was formed by the bromination of **1** at the position *meta*- to the nitro group. The quinoxaline-1,4-dioxide (**25**) was synthesized from benzofuroxan (**24**) bearing methyl ester group and evaluated for its ability to inhibit separase due to its potential bioactivity.⁸ The precursor to **1**, 5-nitrobenzofuroxan **3** and its reduced form **22** were also assayed to determine their inhibition of Separase enzyme.

Scheme 2. Synthesis of derivatives at C₂ position



Compound	R ¹	R ²	Yield (%) from 3
13	CH_3	C_2H_5	63
14	C_2H_5	C_3H_7	21
15	CH_3	C_4H_9	14
16	CH_3	PhCO_2H	25
17	$-(\text{CH}_2)_4^-$		63
18	$-(\text{CH}_2)_5^-$		43

Scheme 3. Other derivatives of scaffold



Inhibition of Separase activity by analogs

As mentioned earlier, **1** exhibits inhibitory activity toward Separase with an IC_{50} of $15 \mu\text{M}$. To determine what structural features are important for Separase inhibition, the derivatives were evaluated for their ability to inhibit

Separase relative to the parent compound **1**. Replacement of the nitro group at the C5 position by other functional groups significantly reduces the inhibitory activity of the derivatives compared to the lead compound **1**. Since, due to their reduction to nitroso functionality, aromatic nitro groups are often problematic in drug development and biological assays the first goal was to replace that group with a pharmacophore equivalent. Unfortunately all versions in which the nitro group has been replaced, including versions with groups that may be considered pharmacophore equivalents for a nitro group, (**4-12**) were less active (Figure 2 A-B). The derivative with a (trifluoromethyl) sulfonyl group substituted for nitro (**8**), inhibits 65.6% Separase enzyme at $100 \mu\text{M}$ concentration, was the most active among those molecules.

As shown in Table 3 the inhibitory activity toward Separase seems to increase with the strength of the electron withdrawing group ($-\text{NO}_2 > -\text{SO}_2\text{CF}_3 > -\text{COOH} > -\text{F} > -\text{Br} > -\text{C}_6\text{H}_5 > -\text{COOCH}_3 > -\text{NH}(\text{C}_4\text{H}_9) > -\text{CF}_3 > -\text{OEt}$). Derivatives that have functional groups with an electron-donating characteristics ($-\text{OEt}, -\text{NH}(\text{C}_4\text{H}_9)$ in (**6** and **10**, respectively) or weakly-electron-withdrawing effects ($-\text{COOH}, -\text{COOCH}_3, -\text{CF}_3, -\text{Br}$, and $-\text{C}_6\text{H}_5$ in **4**, **5**, **7**, **9**, **11** and **12**, respectively) show low inhibitory activity toward Separase. Although derivatives with stronger electron-withdrawing groups such as $-\text{SO}_2\text{CF}_3$ in **8** possesses better biological activity, the Separase inhibition observed with these compounds is still lower than that of **1** with the NO_2 group (Table 3).

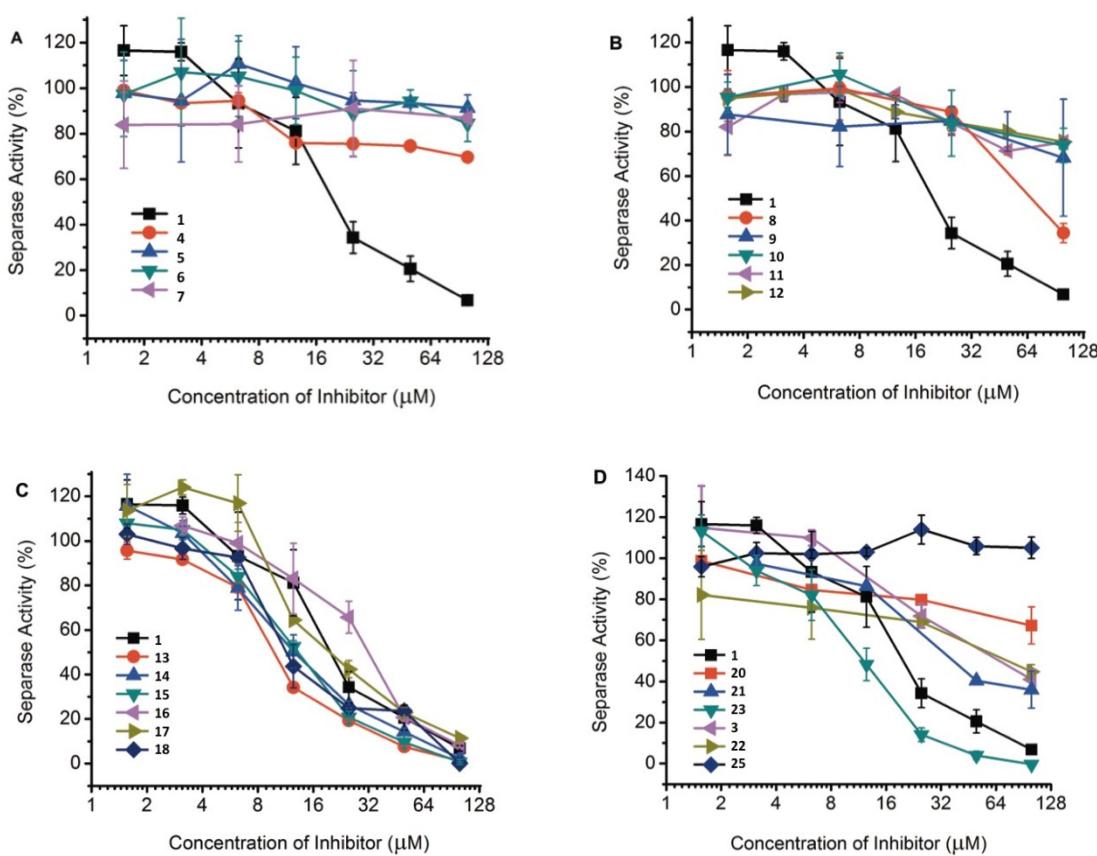


Figure 2. Inhibitory activity of derivatives in Separase enzymatic assay. The inhibitory activity of **1** and its derivatives modified at C₅ (A-B) and C₂ (C) and on the heterocyclic ring (D) was determined using Separase activity assay.

Table 3. Inhibitory activity of Sepin compounds in *in vitro* Separase enzymatic assay shown in Figure 2

Compound (#)	X	R ¹	R ²	IC ₅₀ (μ M) ^d	Inhibitory activity at 100 μ M (%) ^d
Sepin-1 (1)	-NO ₂	-CH ₃	-CH ₃	17.8±2.3	95.9±7.0
(4)	-COOH	-CH ₃	-CH ₃	>100	24.4±8.8
(5)	-COOCH ₃	-CH ₃	-CH ₃	>100	14.6±7.3
(6)	-OC ₂ H ₅	-CH ₃	-CH ₃	>100	21.2±12.3
(7)	-CF ₃	-CH ₃	-CH ₃	>100	8.0±7.0
(8)	-SO ₂ CF ₃	-CH ₃	-CH ₃	50.1±11.4	64.6±3.7
(9)	-F	-CH ₃	-CH ₃	>100	35.4±9.1
(10)	-NH(C ₄ H ₉)	-CH ₃	-CH ₃	>100	40.9±17.3
(11)	-Br	-CH ₃	-CH ₃	>100	24.6±8.4
(12)	-C ₆ H ₅	-CH ₃	-CH ₃	>100	39.3±21.4
(13)	-NO ₂	-CH ₃	-C ₂ H ₅	12.7±1.0	99.6±0.7
(14)	-NO ₂	-C ₂ H ₅	-C ₃ H ₇	14.1±2.9	99.2±1.3
(15)	-NO ₂	-CH ₃	-C ₄ H ₉	14.6±2.1	99.8±0.3
(16)	-NO ₂	-CH ₃	-C ₆ H ₄ (p-COOH)	29.2±10.8	84.1±6.7
(17)	-NO ₂		-(CH ₂) ₄ -	17.1±2.5	96.2±6.6
(18)	-NO ₂		-(CH ₂) ₅ -	16.2±2.3	99.5±0.8
(20) ^a	-F; -OC ₂ H ₅ (o)	-CH ₃	-CH ₃	>100	25.2±22.4
(21) ^a	-NO ₂ ; -F (o)	-CH ₃	-CH ₃	87.2±15.6	60.1±4.6
(23) ^a	-NO ₂ ; -Br (m)	-CH ₃	-CH ₃	12.1±2.6	100
(3) ^b	-NO ₂	-	-	>100	0
(22) ^b	-NO ₂	-	-	>100	43.1±13.2
(25) ^c	-NO ₂	-	-	>100	0

^a The first group is at C₅ position, the position for the second group is given in parenthesis and relative to the first group (o: ortho; m: meta). ^b These molecules are the precursors to Sepini. ^{b,c} See Scheme 3 for chemical structures.

^d The data were from three independent assay.

Replacing the dimethyl group at the C₂ position with different alkyl chains had a moderate effect on the inhibitory activity (Figure 2C). Compound **13** which possesses an ethyl group is 1.4 fold more active than the original compound (Figure 2C, Table 3). Modifying the methyl groups to propyl (**14**), butyl (**15**) or spiro-alkane (**17** and **18**) results in a better or equivalent effect on the inhibition of Separase activity (Figure 2C, Table 3).

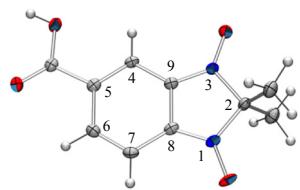
The data suggest that this position could be used to attach a photo-labeling moiety, which would be used to study the mechanism of inhibition of the Separase enzyme. With that in mind, we have synthesized **16**, which has a COOH group at the para position of a phenyl group attached to C₂ of 2H-bezimidazole dioxide skeleton (Scheme 2). This molecule retained moderate activity compared to the lead compound with an IC₅₀ of 29.2 μ M (Figure 2C, Table 3).

Structural modifications of other aromatic positions on the 6-member ring resulted in changes in separase inhibition (Figure 2D, Table 3). The derivatives with fluorine and ethoxy groups at C₅ and C₆ (**20**) exhibit poorer inhibitory activity. While the analog that has a fluorine atom substituted at C₆ (**21**) shows modest activity relative to that of **1**. Interestingly, the derivative with a bromine atom at C₇ (**23**) possesses a 1.5 fold lower IC₅₀ (12.1 μ M) compared to that of the lead structure **1** (Figure 2C, Table 3), and thus

far is the derivative with best activity toward separase enzyme identified.

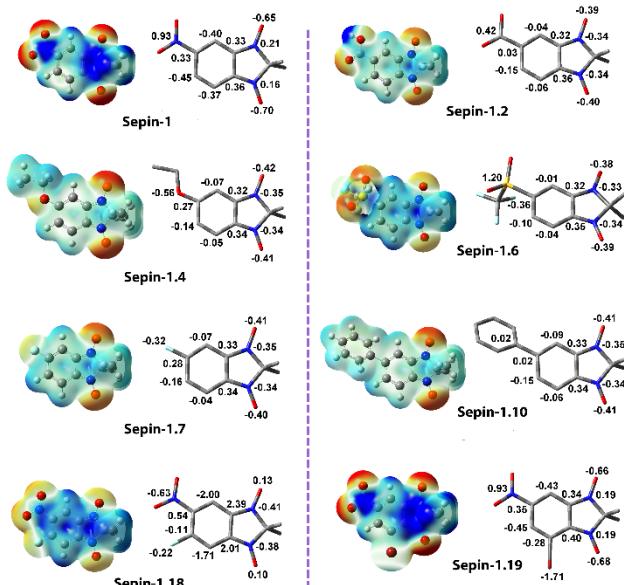
X-ray structural Studies

Due to the inherent simplicity of the NMR spectra of these molecules, the molecular structure of a number of derivatives was confirmed by single-crystal X-ray diffraction. To our knowledge, these structures are the first examples of isolated 2H-benzimidazole-1,3-dioxides derivatives that have been analyzed using single-crystal X-ray diffraction. To date, the only available structure of 2H-benzimidazole-1,3-dioxide characterized by X-ray diffraction was reported by Keller *et al.* in which the molecule is co-crystallized with H[AuCl₄].⁹ For all structures reported in our work, the bond distance of C₄-C₅ and C₆-C₇ bonds in the benzene ring are significantly shorter than the C₉-C₄, C₅-C₆, C₇-C₈, and C₈-C₉ bonds (Table 2). The crystal structures of **4**, **6**, **7**, **9**, **12**, **16**, **17**, **18**, **20** and **23** are all reported in the supplementary material. Interestingly, although this difference in the bond lengths of C-C bonds of the benzene ring was also observed in other 2H-benzimidazole derivatives,¹⁰ there is very little variation for aromatic C-C bonds in the 2H-benzimidazole-1,3-dioxide reported by Keller *et al.*, which may be due to the interaction of the molecule with the [AuCl₄]⁻ anion.⁹

Table 2. Selected Bond Lengths

Bond Length (Å)

C9-C4	1.414(2)
C4-C5	1.364(2)
C5-C6	1.451(2)
C6-C7	1.357(2)
C7-C8	1.417(2)
C8-C9	1.432(4)
N1-O1	1.3048(14)
N2-O2	1.2712(15)
C8-N1	1.3253(19)
C9-N2	1.3326(18)

**Figure 3.** Electrostatic potential surface mapping and Mulliken charges of selected derivatives

Density-functional theory (DFT) calculations have been performed to explore the distribution of electron density on derivatives, with the hope of gaining insight into the biological activity of the tested compounds (Figure 3). The atomic coordinates from the crystal structures of molecules (when available) were used as starting geometry for structural optimization using B3LYP/6-31G* methodology. Figure 3 shows the electrostatic potential surface mapping of selected derivatives and the Mulliken charges of the atoms on the 2*H*-benzimidazole-1,3-dioxide ring skeleton. When comparing the results from calculation and the inhibitory activity toward Separase enzyme, a correlation between the charge on the 5-member ring and the activity of analogs was observed. Derivatives that have more positive charge on the 5-member ring, possess better activity as Separase inhibitors. This charge distribution is dependent on the substituent group on the 6-member ring, particularly at C5. It was also noted that derivatives with more negative charge on the N-O oxygens appear to possess better activity. While there are certainly a number of structural features responsible for the activity observed with the reported molecules, the local electron density appears to play a role.

CONCLUSION

We report the synthesis, structural information and the structure-activity relationship of a novel class of Separase inhibitors based on our lead compound **1**. Various analogs of **1** were synthesized by modifying three main positions on the lead molecule. The bioassay data revealed that, despite our efforts to find an alternative, the NO₂ group at C5 is essential for the activity of analogs in the inhibition of Separase enzyme. Modification at the C2 has a moderate effect on the biological activity of those compounds, suggesting that derivatives from this group could be used to help identify the actual active site in enzyme by photoaffinity labeling studies, which will assist substantially in the search for better inhibitors. Changing the substituents at other positions on 2*H*-benzimidazole-1,3-dioxide or the skeleton of the molecule modifies the inhibition of Separase by those analogs. The derivative with highest activity was found to be the version with bromine-substituted at C7 on skeleton (**23**). DFT calculations illustrate a correlation between the charge on the oxide nitrogen and oxygen atoms and the inhibitory activity toward Separase, suggesting that the oxide moieties on molecule's skeleton may be involved in binding to the Separase enzyme.

While only modest improvement in the activity of the basic scaffold has been achieved this paper clearly illustrates that the nitro group at C-5 appears to be critical for activity. Given that the basic structure does not conform to many of the criteria for medicinal compounds it represents an important tool for use in the study of Separase activity and the potential role of Separase inhibitors in therapy.

EXPERIMENTAL SECTION

Chemistry. General procedures. Unless noted all reagents were purchased from Aldrich or Acros and used without further purification. Thin layer chromatography (TLC) was performed on silica gel 60 F254 pre-coated plates (0.25

mm) from Silicycle and components were visualized by ultraviolet light (254 nm) and/or phosphomolybdic acid or *p*-anisaldehyde stain. Silicycle silica gel 230-400 (particle size 40-63 μm) mesh was used for all column chromatography. All the microwave reactions were performed on Biotage Initiator microwave instrument using Biotage microwave vials (0.5-2 mL, 2-5 mL and 10-20 mL). ^1H and ^{13}C NMR spectra were recorded on JEOL ECX-400 NMR spectrometer (at 400 MHz and 100 MHz respectively) or JEOL ECA-500 NMR spectrometer (at 500 MHz and 125 MHz respectively) in CDCl_3 , $\text{DMSO}-d_6$ and CD_3OD . Chemical shifts were reported in ppm, multiplicities are indicated by s = singlet, d = doublet, t = triplet, q = quartet, sep = septet, dd = doublet of doublet, dt = doublet of triplet, m = multiplet, br = broad resonance. Coupling constants J were reported in Hz. Data for ^{13}C NMR were reported in terms of chemical shift. High resolution mass spectral data were obtained from The University of Texas at Austin, Mass Spectrometry Facility, Austin, TX.

Representative Procedure. To a stirred solution of 1 mmol 5-nitrobenzofuroxan (181 mg) in concentrated sulfuric acid (2 mL) 1.7 mmol 2-propanol (102 mg, 0.13 mL) was added dropwise at room temperature. The reaction mixture was stirred for 2 hours before it was poured into ice-water and extracted with chloroform (3 x 20 mL). The organic layer was dried over MgSO_4 and evaporated. The crude mixture was purified by column chromatography on a silica gel (1% MeOH in CHCl_3), obtained 165 mg of dark violet solid. Yield = 74%. ^1H NMR (400 MHz, CDCl_3) δ 8.18 (s, 1H), 7.58 (d, J = 10.1 Hz, 1H), 7.35 (d, J = 10.1 Hz, 1H), 1.72 (s, 6H). ^{13}C NMR (101 MHz, CDCl_3) δ 149.5, 136.1, 135.7, 123.9, 117.7, 113.9, 99.9, 24.6. HRMS (Cl+) m/z calculated for $\text{C}_9\text{H}_9\text{N}_3\text{O}_4$ m/z 223.0593 ([M] $^+$), found 223.0594 ([M] $^+$).

Separase activity assay. Epitope-tagged Separase was expressed, purified, and activated as described previously.¹¹ The substrate is Rad21-MCA (methyl coumaric acid), which has Rad21 peptide molecule (Asp-Arg-Glu-Ile-Nle-Arg) conjugated to MCA (CPC Scientific, Sunnyvale, CA). The assay was set up using 384-well low-volume black polystyrene plates (Corning Incorporate Life Science, REFERENCE

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Tewksbury, MA). Fifteen microliters of activated separase (~5 ng) were mixed with 5 μl of test compound. The cleavage buffer (CB) used to dilute reagents was 30 mM Hepes-KOH pH 7.7, 50 mM NaCl, 25 mM NaF, 25 mM KCl, 5 mM MgCl₂, 1.5 mM adenosine triphosphate, and 1 mM ethylene glycol tetraacetic acid. The mixture was incubated at room temperature for 0.5 h, and then 5 μl of 0.2 mM substrate Rad21-MCA was added. The positive control contained separase, substrate, and CB without the test compound. The negative control contained substrate and CB only. After the reaction mixture was incubation at 37°C for 3 h, the relative fluorescence intensity (RFI) was measured (without stopping the enzymatic activity) at $\lambda_{\text{ex}} = 390 \text{ nm}/\lambda_{\text{em}} = 450 \text{ nm}$ for MCA released from Rad21-MCA using a SynergyTM 4 Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT).

ASSOCIATED CONTENT

Supporting Information. Single crystal X-ray crystallographic data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Equal Contribution

H.T.D. synthesized all analogs and performed the DFT calculations, structural identification and analysis of them. N.Z. performed all the biological assays. All authors analyzed the data and made decisions on what analogs to synthesized.

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